Studies on blood coagulation factor V

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V. Changes of Molecular Weight Accompanying Activation of Factor V by Thrombin and the Procoagulant Protein of Russell's Viper Venom

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The moiety that converts prothrombin into thrombin (prothrombinase) is thought to be a complex structure built from the protein factors X and V, Ca^{++} ions, and phospholipid. The complex is visualized as a part of a phospholipid micelle to which one molecule of each of two protein factors is adsorbed, one beside the other (Hemker et al., 1967; Jobin and Esnouf, 1967). The binding of factor V is thought to be caused by hydrophobic bonds (Kahn and Hemker, 1970a) whereas Ca^{++} ions are necessary for the binding of factor X (Jobin and Esnouf, 1967; Esnouf and Jobin, 1965; Cole et al., 1965).

It has been shown that factor X, as it occurs in plasma, cannot take part in the complex (Papahadjopoulos et al., 1964). Before it can do so, factor X must be acted upon by either factor VII_a (Deutsch, 1955), the coagulant protein from Russell's viper venom (Esnouf and Williams, 1962), or the intrinsic factor X activator, most probably an enzymatically active complex consisting of factors IX_a, VIII, Ca^{++} ions, and phospholipid (Hemker and Kahn, 1967).

No clear-cut conclusions have been reached as to the form in which factor V figures in the complex. Some hold the view that activation of factor V is not necessary (Esnouf and Jobin, 1967), whereas others show that factor V can be activated by thrombin or Russell's viper venom (Macfarlane, 1961; Hussain and Newcomb, 1963; Ware et al., 1947; Hjort, 1957; Williams and Esnouf, 1962).

Materials and Methods

Bovine factor V was prepared from bovine plasma (18 mMoles in Na-oxalate) according to Papahadjopoulos (Papahadjopoulos et al., 1964). The four preparations used contained 2.0–3.0 u/ml of factor V and 3.2–4.8 mg protein/ml, 1 unit of a coagulation factor being defined as the amount contained in 1 ml of fresh human reference plasma. No other coagulation factor activities were detectable in these preparations. Protein determination was carried out according to Folin and Ciocalteau.

Human factor V was prepared as described elsewhere (Kahn and Hemker, 1970b). The six preparations used contained 0.5–0.75 u/ml of factor V and 1.2–2.1 mg protein/ml. No other coagulation factors could be demonstrated in these preparations.

In the activation experiments the factor V preparation was incubated at 37° C with the amount of Russell's viper venom or thrombin indicated, with or without 2 μg of hirudin per ml (Sigma, St. Louis, grade A). The portions used for gel filtration experiments were incubated until no

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further rise in activity was observed. Russell's viper venom (R. V. V.) was obtained from Burroughs Wellcome Ltd. The purified procoagulant protein fraction from Russell's viper venom was a kind gift of Dr. M. P. Esnouf, and was prepared according to Williams and Esnouf (1962). The thrombin was bovine thrombin (Roche) or a purified human thrombin prepared according to Strässle (1963). Gel filtration was carried out at 4° C on 2.5 × 100 cm Pharmacia columns filled with G 200 Sephadex or biogel P 300 polyacrylamide gel; U. V. adsorption was monitored with a L. K. B. Uvicord I. The fractions were collected in a L. K. B. Radirac fraction collector.

The medium for gel filtration was 0.05 M $MgSO_4$ in 0.05 M Tris-lactate (pH 7.5) containing 50% (v/v) glycerol, unless otherwise indicated.

Molecular weights were estimated from the elution volumes as described by Andrews (1965) and Whittaker (1953). Yeast alcohol dehydrogenase (EC 1.1.1.1.) was used as a reference of known molecular weight (M = 126,000). Its activity was measured according to Bergmeyer (1962). Dextran-blue was used to measure the void volume of the columns. To determine an elution volume from the activity curves found in the chromatographic experiments a best fit was simulated to these curves with aid of a Dupont 310 curve resolver. The localization of the top of the gaussian curve that gave the main contribution (≥70%) to the simulated activity curve was thought to indicate the elution volume.

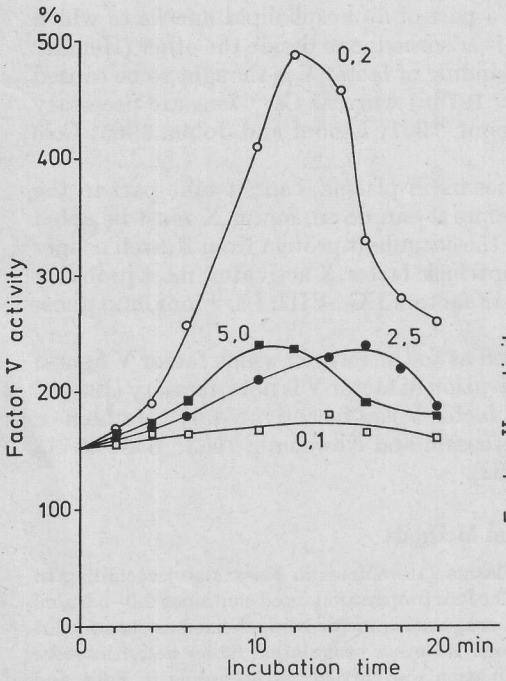

Fig. 1

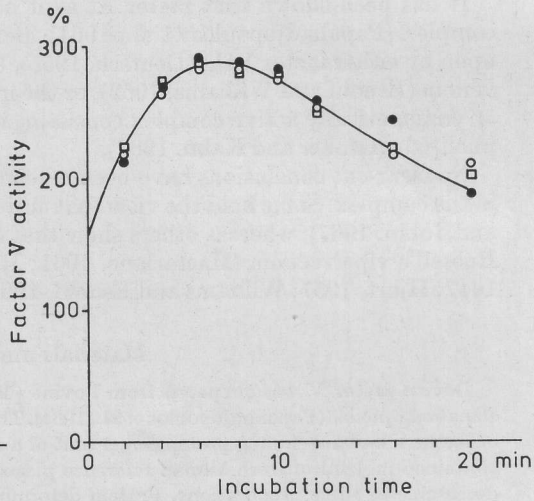

Fig. 2

Fig. 1. Activation of bovine factor V by the procoagulant protein from Russell's viper venom. The starting material was a preparation of purified bovine factor V with an activity of 152% of our standard plasma. 2 μg of hirudin was added. ○———○ activation by 0.2 μg/ml purified R. V. V.; □———□ activation by 0.1 μg/ml crude R. V. V.; ●———● activation by 2.5 μg/ml crude R. V. V.; ■———■ activation by 5.0 μg/ml crude R. V. V.

Fig. 2. Activation of bovine factor V by Russell's viper venom in the presence of hirudin and Ca^{++}. The starting material was a preparation of purified bovine factor V with an activity of 152% of our standard plasma. Incubation mixture as in Fig. 1, except for the modifications indicated below: □———□ 2 μg/ml hirudin; ●———● 2 μg/ml hirudin and 10 mM Ca^{++}; ○———○ no additions.

Experimental

Russell's viper venom causes a marked activation of bovine factor V (Fig. 1). This is independent of the presence of Ca^{++} ions or hirudin (Fig. 2). From Fig. 1 it can be seen that a preparation from Russell's viper venom purified according to Esnouf and

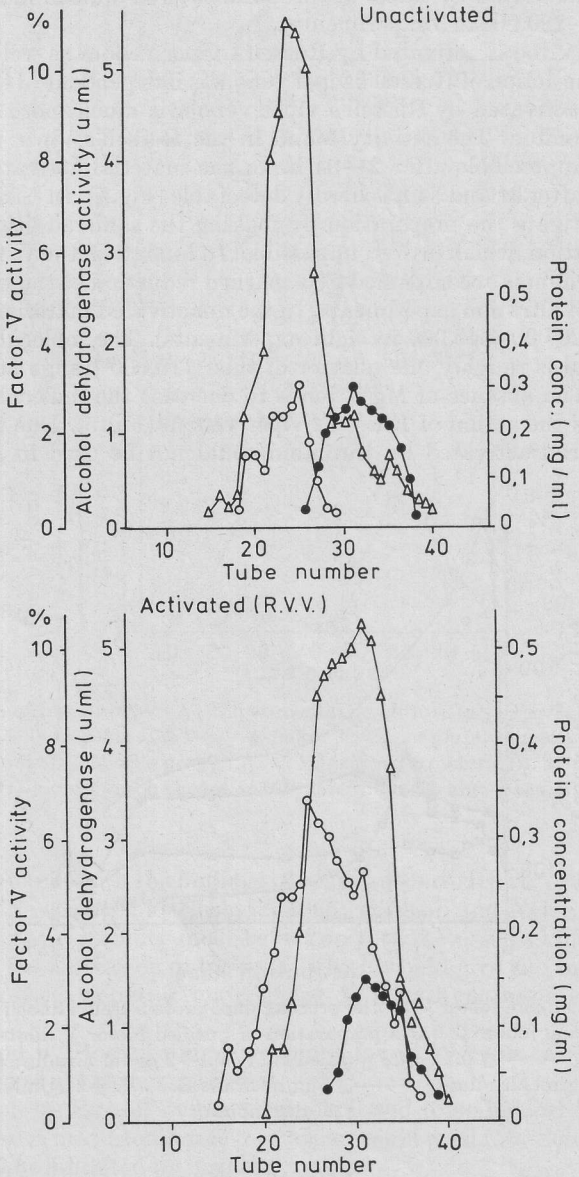

Fig. 3. Gel filtration of bovine factor V. ○———○ protein; ●———● alcohol dehydrogenase; △———△ factor V. Column: G 200 Sephadex. The activated sample was treated with 0.1 μg of purified Russell's viper venom.

Williams (1962) is also highly active as a factor V activator. Gel filtration experiments show (Fig. 3) that the action of Russell's viper venom on bovine factor V results in an increase of the elution volume. When the elution volume is taken as an indication of the molecular weight it can be calculated that the molecular weight drops from 400,000 (370,000–430,000 in six experiments) to 195,000 (180,000–230,000 in 6 experiments). Bovine factor V activated by traces of thrombin showed a mean molecular weight of 180,000 (160,000–220,000 in 5 experiments).

Human factor V too is activated by Russell's viper venom as well as by thrombin (Fig. 4). Again, the action of Russell's viper venom is independent of Ca^{++} and hirudin. Human factor V activated by Russell's viper venom is much more stable than that activated by thrombin. The activity found in the Russell's viper venom activated material is still appreciable after 21–34 h. In the material activated by thrombin, residual activity after 21 and 34 h is hardly detectable (Fig. 4). In parallel experiments we tried to re-activate the preparation by making the same addition as that which started the activation, which proved impossible. In human factor V, the activation by Russell's viper venom is accompanied by a marked reduction of the molecular weight, as judged from gel filtration experiments. In the unactivated material, M equals about 480,000 (range 420,000–540,000 in eight experiments). The molecular weight of the activated material is roughly one quarter of this: 110,000 (range 90,000–130,000 in 5 experiments). The absence of Mg^{++} tends to decrease the molecular weight in the same way as does the action of Russell's viper venom (Fig. 5). Due to its instability, the human material activated by thrombin could not be used in gel filtration experiments.

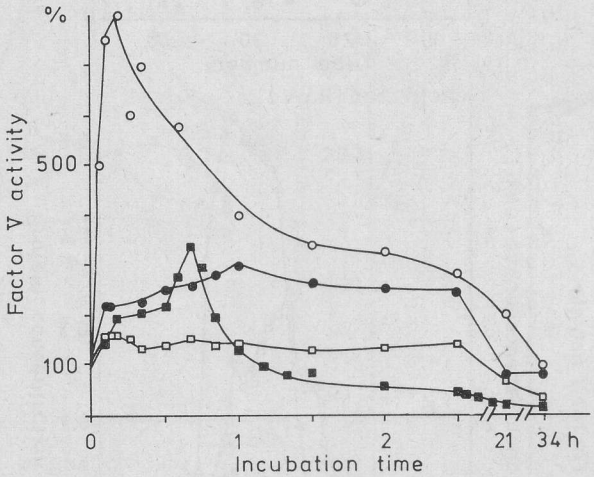

Fig. 4. Activation of human factor V by the procoagulant protein from Russell's viper venom and thrombin. The starting material was a preparation of purified factor V diluted so as to contain 100% of factor V. ○———○ 0.2 μg/ml purified R. V. V. +2 μg/ml hirudin; ●———● 2 μg/ml crude R. V. V. +2 μg/ml hirudin; □———□ 5 μg/ml crude R. V. V. +2 μg/ml hirudin; ■———■ 5 u/ml thrombin.

Discussion

From the results shown in Figs. 1, 2 and 4, it may be concluded that both Russell's viper venom and thrombin activate human as well as bovine factor V.

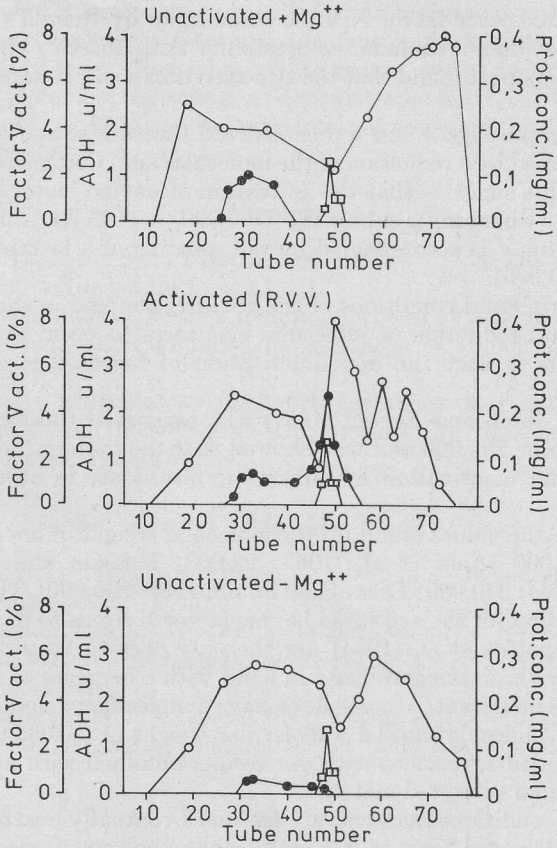

Fig. 5. Gel filtration of human factor V. Experimental conditions as in Fig. 3. ○———○ protein; □———□ alcohol dehydrogenase; ●———● factor V. Upper graph: unactivated material in the presence of 0.05 M $MgSO_4$. Middle graph: R. V. V. activated material in the presence of 0.05 M $MgSO_4$. Lower graph: unactivated material in the absence of $MgSO_4$.

This is in accordance with the findings of other authors (Hjort, 1957; Bergsagel and Nockolds, 1965; Newcomb and Hoshida, 1965; Hussain and Newcomb, 1963; Ware et al., 1947). Still the possibility must be left open that Russell's viper venom expresses its action via the activation of factor X, which would give rise to the formation of thrombin, which then would activate factor V. This hypothesis must be rejected, because:

1. the activation by Russell's viper venom is found in media in which neither factor X nor factor II could be demonstrated;

2. the activation by Russell's viper venom is found to be independent of Ca^{++} ions and hirudin, whereas prothrombinase formation would need Ca^{++} ions and any thrombin formed would be inhibited by hirudin.

Russell's viper venom therefore seems to have two procoagulant activities. In the first place, it activates factor X, as proven by Macfarlane (1961); secondly, its activates factor V. In this context it must be mentioned that the experiments of Macfarlane

unequivocally demonstrate factor X to be acted upon by Russell's viper venom. On the other hand, they do not exclude the possibility that Russell's viper venom acts on factor V as well. Rapaport found that the two activities could be separated (Rapaport, 1968).

The results given in Figs. 3 and 5 demonstrate that activation by Russell's viper venom is accompanied by a reduction of the molecular size. Comparison of the estimated molecular weights suggests that the activation of bovine factor V is accompanied by the splitting of a dimer in its subunits (~400,000 → ~195,000) and that the activation of human factor V is accompanied by the splitting of a tetramer in its subunits (~480,000 → ~110,000).

Under the experimental conditions of the gel filtration and in the absence of Mg^{++} ions, activation and reduction of molecular size seem to occur spontaneously. The conclusion presents it that the depolimerization of factor V is not necessarily an enzymatic process.

The findings of Jobin and Esnouf (1967) who estimated the molecular weight of bovine factor V to be 290,000 and who showed that the material was liable to reversible activation and inactivation by enzymatic means are in accordance with this conclusion.

In the literature the values found for the molecular weight of bovine factor V range from 98,000–290,000 (Aoki et al., 1963; 98,000; Hussain and Newcomb, 1963: 180,000; Lewis, 1964: 270,000; Esnouf and Jobin, 1967: 290,000). The value of around 195,000 that we found for the activated bovine factor V seems to fit somewhere in this range. Papahadjopoulos et al. (1964) are the only ones to state that activation of bovine factor V by thrombin goes hand in hand with a decrease of molecular weight; they estimated the unactivated molecule to have a molecular weight of above 400,000, and the activated molecule showed a molecular weight of >200,000 in their experiments. This is in good agreement with our results obtained with both thrombin and Russell's viper venom activated material.

Apparently the conditions that activate factor V eventually lead to its inactivation; presumably the activated form is less stable than the unactivated one. The active form of human factor V obtained by the action of Russell's viper venom is much more stable than that obtained by thrombin. As yet, no explanation for this phenomenon can be given. It is noteworthy that in our medium we did not observe an interaction between factor V and G 200 Sephadex as reported by Esnouf and Jobin (1967).

The experiments reported here suggest that the reactions in which factor V takes part in their simplest form can be described by the following equations:

$$\overset{1}{\underset{}{}} \qquad \overset{2}{\underset{}{}}$$
$$P \rightleftarrows 4(2)A \rightarrow 4(2)A^1$$

P denotes a preactive or partially active state of the molecule, A and A^1 the active state. The figures in brackets refer to bovine material, the others to human material. We do not know whether the P form has any activity of its own, because it can be activated during the assay procedure.

Reaction 1 refers to the spontaneous non-enzymatic activation. This reaction may be reversible. Reaction 2 is an enzymatic step brought about by Russell's viper venom or thrombin. This reaction presumably is irreversible. The A^1 form and possibly the A form too, are less table than the P form. The greater instability of the A^1 form obtained with thrombin as compared to that obtained with Russell's viper venom may mean that reaction A differs for the two enzymes, or that thrombin has a further inactivating action on A^1.

Changes in the elution volume accompanied by an activation would also occur when factor V started to complex with factor X_a or phospholipid, as it does during the formation of prothrombinase. Such changes, however, would point to an *increase* of molecular weight instead of a decrease as demonstrated here. The high concentration of Mg^{++} ions in the gel filtration experiments moreover inhibits any complexation (Esnouf and Jobin, 1967).

Summary

It is confirmed that a protein from Russell's viper venom has a direct activating action on human as well as bovine factor V.

Unactivated human factor V has a molecular weight as estimated by gel filtration of ~410,000, after activation the molecular weight is ~110,000, thus suggesting the dissociation of a tetramer.

The approximative molecular weight figures for unactivated and activated bovine factor V are 400,000 and 195,000, resp. This suggests the dissociation into a dimer accompanying the process of activation.

Résumé

On confirme qu'une protéine du venin de la vipère de Russel active de façon directe le facteur V humain et bovin.

Le facteur V humain non activé a un poids moléculaire de ~480000 estimé par gel filtration, après activation le poids moléculaire est de 110000 environ ce qui suggère la dissociation d'un tétramère.

Les poids moléculaires pour le facteur V bovin non activé et activé sont de 400000 et 195000. Ce résultat suggère une dissociation en dimères pendant l'activation.

Zusammenfassung

Es wird bestätigt, daß Russells-Viperngift eine direkte Aktivierung des Faktors V bei Mensch und Rind bewirkt.

Nicht aktivierter menschlicher Faktor V hat das Molekulargewicht, bestimmt durch Gelfiltration, von ungefähr 480000. Nach der Aktivierung beträgt es 110000, was für eine Dissoziierung eines Tetramers spricht.

Die ungefähren Molekulargewichte des nicht aktivierten und des aktivierten Faktors V vom Rind betragen 400000 und 195000. Dies spricht für eine Dissoziation in ein Dimer während des Aktivierungsvorganges.

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